Agrobacterium mediated transformation of black pepper using sequences from *Cucumber mosaic virus* and *Piper yellow mottle virus*

PhD Final Synopsis

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1. INTRODUCTION

Black pepper (*Piper nigrum* L.*)* is a flowering vine of family Piperaceae grown for its berries. It is believed to have originated in the sub mountainous tracts of the Western Ghats of India (Rahiman et al. 1979). It has been one of the most priced spices cultivated since millennia. Black pepper is widely used in cooking, processing of food and perfumery (Philip et al. 1992; Bhat et al. 1995). It has multidimensional medicinal properties (Kumar et al. 2007; Scott et al. 2008) and anti microbial properties (Dorman and Deans 2000; Umit et al. 2009). It is chiefly cultivated in the tropical countries of the world such as India, Sri Lanka, Vietnam, Malaysia, Indonesia, Brazil and West Indies. Black pepper is infested by fungal, viral and phytoplasmal pathogens. Infection caused by viruses and phytoplasma are difficult to control and are transferred by vegetative propagation. Latent pathogens especially viruses often result in loss of plant production and a poor quality product (Phillip et al. 1992). Among the biotic stresses, stunted disease caused by viruses occupies third position in the form of loss they cause on black pepper. Association of two viruses namely *Cucumber mosaic virus* (CMV) and *Piper yellow mottle virus* (PYMoV) were reported with the disease (Lockhart et al. 1997; de Silva et al. 2002; Bhat et al. 2003; 2005a; Hareesh and Bhat 2008). CMV is the type species of the genus, *Cucumovirus* in the family Bromoviridae. Its genome consists of three plus single stranded RNAs and a fourth sub genomic RNA. It has a broad host range and is readily transmitted in a non persistent manner by more than 75 species of aphids

(Palukaitis et al. 1992). PYMoV is a member of the genus *Badnavirus* in the family Caulimoviridae. Badnaviruses are plant pararetroviruses with non enveloped bacilliform particles having a dimension of 30 x 130-150 nm that contain a genome consisting of a circular, relaxed 7.5 – 8.0 kb double stranded DNA molecules (Lockhart 1990; Bouhida et al. 1993; Qu et al. 1991). They have a narrow host range and shown to be transmitted through mealy bug (*Planococcus citri* and *Ferrisia virgata*) and black pepper lace bug (*Diconocoris distanti). (*de silva et al. 2002; Bhat et al. 2003; Bhat et al. 2005 b).

The primary spread of viruses is through the use of infected cuttings for planting while secondary spread is through insect vectors in the field. None of the cultivars of black pepper grown is resistant to viruses. Since there is no effective chemical to control viruses, it is necessary to impart resistance against viruses in agronomically superior cultivars. With no available source of resistance, the scope of improvement of black pepper by conventional breeding is very remote; hence genetic transformation of black pepper with foreign genes could be useful to impart resistance.

Genetic engineering for virus resistance is one of the major success stories in plant biotechnology. Transgenic approaches have proven to be able to produce durable and safe virus resistance in the field, enabling the production of crops that would otherwise were not been possible (Fuchs and Gonsalves 2007). Genes derived from pathogen itself can be used to impart resistance to that specific pathogen or closely related pathogen, usually referred as pathogen derived resistance (PDR). It has enormous application in developing virus resistance as plant derived virus resistance genes are often not available. Transgenic plants expressing coat protein or other viral sequences were shown to confer resistance against virus in many virus-host combinations. Similar approach can be used to transform existing cultivars of black pepper to get resistant plants through transformation using viral sequences. Coat protein gene of CMV and a portion of open reading frame III (ORF III) of PYMoV infecting black pepper have been cloned and their nucleotide sequence have been determined (Bhat et al. 2005a; Hareesh and Bhat 2008). With this in view the present study was conducted with following objectives

1. To develop an *Agrobacterium* mediated transformation system for black pepper.

2. To develop gene constructs using *Cucumber mosaic virus* (CMV) and *Piper yellow mottle virus* (PYMoV) sequences in plant transformation vector pBI121.

3. Development of transgenic lines of black pepper using viral constructs and plantlet regeneration.

4. Screening and analysis of transformants.

2. MATERIALS AND METHODS

2.1 Development of an *Agrobacterium* mediated transformation system for black pepper

Selection of suitable explant for transformation

Explants and their preparation

Five types of explants *i.e.* leaf, internode and petiole from *in vitro* propagated black pepper plants of cv. Karimunda, embryo along with surrounding micropylar tissue and embryogenic mass from mature seeds of black pepper cv. Karimunda were used as explants.

Leaf, internode and petiole

Leaf, internode and petiole were obtained from contamination free *in vitro* grown mother cultures. Mother cultures of cv. Karimunda were established by the method described below.

Nodal explants were collected from black pepper cv. Karimunda. These explants were treated with 0.2% bavistin for 15-20 min and washed thoroughly with sterile distilled water to remove traces of fungicide. Explants were then treated with 0.1% mercuric chloride for 4-6 min. After washing with sterile distilled water for 3-4 times, they were directly inoculated in to basal woody plant medium (WPM) (Mc Cowen et al. 1979) and incubated at 26 ± 2^0C at light intensity of 1500 lux and 10 h photoperiod. Cultures were periodically observed and contaminated ones were removed. Fully grown cultures were maintained by regular subculturing. Explants such as leaf segments, nodal region and petiole taken from *in vitro* grown plants were used for standardizing the regeneration protocol by using Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt 1972), and Murashige and Skoog (MS)

medium (Murashige and Skoog 1962) having different combinations of BAP, TDZ, kinetin and NAA.

Embryo along with surrounding micropylar tissue

Direct somatic embryogenesis from embryo along with surrounding micropylar tissue of black pepper

This was carried out by slightly modifying the protocol described by Nair and Gupta (2006) where scooped out embryo along with surrounding micropylar tissue was used instead of mature seeds as source of explant. Explants were prepared as follows: Mature seeds of black pepper (cv. Karimunda*)* collected from plants grown in the germplasm repository at Indian Institute of Spices Research, Calicut were used as source materials for establishing primary somatic embryogenic cultures. Seeds were soaked in tap water for overnight and outer fleshy mesocarp of the seed was removed by slight rubbing before surface sterilization. Seeds were surface sterilized with 0.1% mercuric chloride solution for 5 min, followed by repeated washings (3-4 times) with sterile double distilled water. Seeds were allowed to dry on sterile filter paper in laminar air flow for 30 min. Then the embryo along with micropylar tissue was dissected out aseptically with a sterile scalpel. The dissected tissues were cultured on agar gelled full-strength, plant growth regulator (PGR) free SH medium containing 3% (w/v) sucrose under darkness for 90-120 days. The pH of the medium was adjusted to 5.9 before autoclaving.

Induction of cyclic secondary somatic embryogenesis and establishment of embryogenic mass

Primary somatic embryo clumps having pre-globular to torpedo shaped embryos (4–6 visible embryos per seed) were carefully detached using the tip of a sterile scalpel blade and inoculated on full strength, PGR free SH medium containing 1.5% sucrose and gelled with 0.8% agar and incubated in complete darkness for the production of embryogenic mass. The pH of the medium was adjusted to 5.9 before autoclaving. Embryogenic mass was maintained by regular subculturing at 30 days interval in the same medium.

Conversion of embryogenic mass in to fully developed plantlets and its genetic fidelity analysis by RAPD markers

Conversion of embryogenic mass into plantlets was done as per the protocol of Nair & Gupta (2006). Ten randomly selected plants were used for genetic fidelity analysis. Total genomic DNA isolated from 100 mg leaves of these plants following the protocol described by (Hareesh and Bhat 2008). This was used for genetic fidelity analysis using RAPD markers. Twenty three 10-mer primers (Sigma Genosys Bangalore, India), having GC concentration of 60–70%, were used for RAPD amplifications.

Determination of phytotoxic levels of bactericidal and selective antibiotics
Antibiotics used

Antibiotics used in the experiment were cefotaxime, carbenicillin and kanamycin. All the three antibiotics were dissolved in distilled water, filter sterilized, and stored in sterile centrifuge tubes at –20° C.

Effect of carbenicillin and cefotaxime on embryo (along with micropylar layer) and embryogenic mass

On embryo along with micropylar tissue

Embryo along with micropylar tissue was treated with five different concentrations (25, 50, 100, 250, 500 µg/ml) of cefotaxime and carbenicillin to test its effect on somatic embryo induction.

On embryogenic mass

Embryogenic mass was treated with different concentrations of antibiotics to test their effect on somatic embryo proliferation and growth of plantlets. Five different concentrations (25, 50, 100, 250, 500µg/ml) of cefotaxime and carbenicillin were used.

Optimization of minimum lethal concentration of kanamycin for the selection of transformants

Embryo along with micropylar tissue, embryogenic mass and fully developed plantlets were used as explants to determine the optimum concentration of kanamycin

required for the selection of transformants. The different concentrations of kanamycin used were 10, 25, 50, 100, 200 µg/ml.

A. tumefaciens strain and plasmid

The *A. tumefaciens* strain EHA 105 (Hood et al. 1993) harboring a binary vector, pBI121 was used in the present study.

Optimization of carbenicillin and cefotaxime concentration to control *Agrobacterium* over growth

In order to find out the optimum concentration of antibiotics (cefotaxime and carbenicillin) to control *Agrobacterium* over growth from transformed tissue, different concentrations (25, 50, 100, 250, 500 µg/ml) of cefotaxime and carbenicillin were tested. The co-cultured embryogenic mass was transferred to selection medium (SH basal +1.5% sucrose + antibiotics of respective concentration), and the plates were cultured for 3–5 weeks under darkness. *Agrobacterium* over growth in explants were recorded.

Transformation using GUS construct and analysis of transformants

Transformation and regeneration

About one g of embryogenic mass was infected (with intermittent vigorous shaking for 2 h) with $1/5^{th}$ diluted 18 h grown culture (OD_{600}-1.5, OD_{600}=1.0 corresponds to 1 $X10^8$ cells/ ml) of *A. tumefaciens* EHA 105 containing the binary plasmid pBI121. The co-cultured embryogenic mass was transferred to selection medium (SH basal +1.5% sucrose +100 µg/ml cefotaxime +25 µg/ml kanamycin), and the plates were cultured for 3–5 weeks under darkness. Some vigorous growing points were observed in the first round of selection and growing points were removed onto the same medium with a higher kanamycin concentration (50 µg/ml). The proliferated embryogenic mass was then transferred to basal SH (liquid) with 3% sucrose for further development of embryos into fully developed plantlets. Fully developed plantlets were transferred individually to WPM with 3% sucrose, 0.8% agar, 100 µg/ml kanamycin and 0.2% charcoal. The non transformed plantlets started bleaching within 15 days, the non bleached plants till 30 days were transferred to fresh medium of same composition but without kanamycin for another 30 days. Surviving plants were hardened in sterile potting mixture (sand, soil, farmyard manure-1:1:1) in the green house and screened for the presence of transgene by PCR, dot blot and Southern hybridization.

Confirmation of transgenicity

Assay for GUS activity

The GUS expression in *Agrobacterium* treated embryogenic mass after one month was determined histochemically by using 'β-Glucuronidase (GUS) Reporter Gene Staining Kit' (Sigma, Missouri, USA) by following the manufacturer's instructions. The possibility of endogenous GUS expression was tested by subjecting uninfected somatic embryos and tissues to the histochemical GUS assay. GUS expression was visually observed and photographed under a light stereo-zoom microscope (Nikon HF II)

PCR analysis of transformed plants

DNA isolated from young unfolded leaves (100mg) of putative transformed and untransformed control plants using a CTAB procedure (Hareesh and Bhat 2008) was subjected to PCR using primers specific to NPT II and GUS genes.

Dot blot hybridization to determine the presence of transgene

Total genomic DNA isolated from 100 mg leaves of 6 month old hardened plants were used for dot blot hybridization. Probe preparation, hybridization and detection were performed using 'DIG High Prime DNA Labelling and Detection Starter Kit II' (Roche Applied Science, IN, USA) following the manufacturer's instructions.

Southern hybridization of transformed plants

Genomic DNA isolated from the leaf tissue (1g) by CTAB method (Ausbel et al. 1995) was subjected to Southern blot hybridization analysis using standard protocols (Sambrook and Russell 2001). Fifteen µg of sample DNA was restricted and size-fractionated in a 0.7% agarose gel by electrophoresis, subsequently transferred to a nylon membrane (Porablot NY plus, Macherey Nagel, Germany) by capillary method. A 980 bp DNA fragment corresponding to the kanamycin gene was labeled with digoxigenin and used as a probe for hybridization according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Plasmid vector pBI121, after digestion with same restrtiction enzyme (250 ng) was used as a positive control while genomic DNA from a non-transformed plant served as a negative control. Hybridization procedures and incubation with chemiluminescence substrate CSPD were carried out in accordance with the manufacturer's

instructions. The tracks of chemiluminescence were captured, developed and fixed in X-ray film (Kodak) as per standard procedures.

2.2 Development of gene constructs using CMV and PYMoV sequences in plant transformation vectors

ORF III region of *Piper yellow mottle virus* (PYMoV) in sense and antisense orientation

ORF III region of PYMoV infecting black pepper was originally cloned in pPCRScriptAmpSK(+) vector in sense orientation (Hareesh and Bhat 2008) available at The Division of Crop Protection , Indian Institute of Spices Research, Calicut was used in the present study. The recombinant cloning vector was designated as pPCRScriptAmpSK(+)ORF III. The cloned region of ORF III harboured a *Bam*H1 site at 409 bases from its 5´ end. So a 409 bp fragment was released on single digestion with *Bam*H1. Plant transformation vector pBI121 was used as it possessed same restriction site where 409 bp of ORF III region could be cloned in both sense and antisense orientation under CaMV 35S promoter. Competent *E. coli* (DH 5α) was then transformed using standard procedures. Positive recombinants harboring ORF III region were identified by PCR and restriction analysis. Plasmid DNA isolated from positive recombinants was then subjected to PCR analysis to identify sense and antisense oriented clones. Each of the identified sense and antisense clones were then mobilized into *A. tumefaciens* strain EHA 105 by triparental mating with the help of helper plasmid pRK 2014.

Cucumber mosaic virus (CMV) coat protein in sense orientation

Coat protein (CP) of gene (657 bp) of *Cucumber mosaic virus* (CMV) infecting black pepper originally cloned in pPCRScriptAmpSK(+) vector in sense orientation (Bhat et al. 2005b) available at Division of Crop Protection, Indian Institute of Spices Research, Calicut was used in the present study. The recombinant cloning vector was designated as pPCRScriptAmpSK(+)CMVCP. The CP gene was released from the vector by double digestion with restriction enzymes *Sac*1 and *Bam*H1 and sub cloned in to pBI121 at *Bam*H1 – *Sac*I site Recombinant nature of plasmid was identified by PCR and restriction analysis. This was then mobilized into *Agrobacterium tumefaciens* strain EHA 105 by triparental mating with the help of helper plasmid pRK 2014.

2.3 Transformation of embryogenic mass with PYMoV sense (pBI121PYMoVS) and antisense (pBI121PYMoVAS) constructs and screening of tranformants

The PYMoV ORF III constructs containing 409 bp region in sense (pBI 121 PYMoVS) and antisense (pBI 121 PYMoVAS) orientation in *A. tumefaciens* EHA 105 were used for transformation of embryogenic mass. Transformation procedure was same as mentioned for GUS construct. Surviving plants were screened for the presence of transgene by PCR, dot blot and Southern hybridization.

PCR was performed using two sets of primers. One set primer was specific for kanamycin region while the other set was CaMV 35S promoter region (as forward primer) and reverse primer of ORF III region (as reverse primer) in the case of pBI121PYMoVS transformed plants, CaMV35S promoter region (as forward primer) and forward primer of ORF III region (as reverse primer) were used to test transformed plants obtained using pBI121PYMoVAS construct. PCR positive plants were subjected to dot blot assay and plants that gave intense spots in dot blot assay were taken for southern hybridization.

2.4 Transformation of embryogenic mass with CMVCP sense (pBI121CMVCPS) construct and analysis of transformants

CMV CP construct in sense (pBI121CMVS) orientation in *A. tumefaciens* EHA 105 were used for transformation. Transformation procedure was same as mentioned for GUS construct. Surviving plants were screened for the presence of transgene by PCR, dot blot and Southern hybridization. Surviving plants were screened for the presence of transgene by PCR, dot blot and Southern hybridization.

PCR was performed using two sets of primers. One set primer was kanamycin specific and the other set was primer designed for the CaMV 35S promoter region as forward primer and reverse primer of CMVCP as reverse primer. Primers designed for CaMV 35 S promoter region (forward) and 3' end of CMVCP transgene (reverse) were used in the amplification which would give an amplicon of 780 bp in transformed plants. All PCR positive plants were subjected to dot blot assay and plants that gave intense spots in dot blot assay were taken for southern hybridization.

3. RESULTS

3.1 Development of an *Agrobacterium* mediated transformation system for black pepper

Selection of suitable explant for transformation

Five types of black pepper explants (leaf, internode and petiole, embryo along with micropylar region and embryogenic mass) were tested for their regeneration ability in MS and SH media with different hormonal combinations. Among them only embryo along with micropylar region and embryogenic mass gave successful regeneration while leaf, internode and petiole explants failed to give regeneration. Embryo along with the surrounding micropylar tissue was able to exhibit primary somatic embryogenesis but with low frequency (14%) with an average of 4-6 somatic embryo per explant. But embryogenic mass obtained from primary somatic embryo through cyclic secondary embryogenesis was able to give high frequency regeneration of about 75 – 100 plantlets from every 250 mg of embryogenic mass. About 90% of the plantlets rooted without any additional treatment. SH medium without any of the plant growth hormone was used for the entire life cycle of the plants. All regenerated plants showed normal growth and development with uniform appearance like any normal black pepper plants.

Conversion of embryogenic mass in to fully developed plantlets and its genetic fidelity analysis by RAPD markers

A total of 150 scorable bands were analyzed by 23 random primers with an average of 6.52 bands per primer and all the 150 markers were monomorphic across all the plants tested. This demonstrates that there was no genetic variation among the progeny and to the parent plant from which they were originated.

Determination of phytotoxic levels of bactericidal and selective antibiotics

Effect of carbenicillin and cefotaxime

On embryo along with micropylar tissue

Carbenicillin at all tested concentrations and cefotaxime >100 µg/ml inhibited primary somatic embryo induction. About 2-3% of embryos showed primary somatic embryo induction in presence of cefotaxime concentration ranging from 25-100 µg/ml while in control 14% of embryos showed primary somatic embryo induction.

On embryogenic mass

The effects of carbenicillin and cefotaxime on embryogenic mass was evaluated based on the number and growth of plantlets produced after 45 days of culturing of embryogenic mass on SH medium containing different antibiotic concentrations. Of the two antibiotics, carbenicillin was more inhibitory to number of plantlets produced. In general addition of carbenillicin at all tested concentrations significantly reduced number of plantlets produced compared to corresponding concentrations of cefotaxime. Maximum (108.3) and minimum (8.66) number of plantlets were produced in treatments containing 100 µg/ml of cefotaxime and 500 µg/ml of carbenillicin respectively. Results clearly showed that cefotaxime at 100 µg/ml produced significantly higher number of plantlets (108.3) compared to control (62). In both antibiotics, number of plantlets produced were increased with increasing concentration of antibiotics up to 100 µg/ml, thereafter decrease in number plantlets was seen.

With regard to plantlet growth, among treatments, maximum growth (3.233 cm) was seen in embryogenic mass was treated with carbenellicin at 100 µg/ml which was on par with control (3.5 cm). Minimum growth of plantlets (0.8 cm) was recorded with carbenicillin at 500 µg/ml. In general, increase in antibiotic concentration up to 100 µg/ml increased plant growth while further increase >100 µg/ml resulted in decreased growth. Both antibiotics at all concentrations (except carbenicillin at 100 µg/ml) retarded plantlet growth compared to control

Optimization of minimum lethal concentration of kanamycin for the selection of transformants

Results showed that kanamycin at all concentration tested inhibited the germination of zygotic embryo and primary somatic embryo induction. But embryogenic mass treated with kanamycin at lower concentrations (10 and 25 µg/ml) survived although there was significant reduction in number of somatic embryos produced. At 50 µg/ml of kanamycin, blackening around embryogenic mass, at 100 µg/ml complete blackening of embryogenic mass was seen and only a few embryos produced (3.3). At 200 µg/ml of kanamycin, complete blackening of embryogenic mass was observed and only 0.6 embryos were produced.

Impact of kanamycin on fully developed plantlets was measured on the basis of number of bleached plants within 30 days of time. Results showed that plantlets treated with

kanamycin up to 50 µg/ml, survived without bleaching. Plantlets treated with 100 and 200 µg/ml of kanamycin started bleaching within 15 days and after 30 days 60% and 100% of plantlets were bleached respectively.

Results indicated that kanamycin at 200 µg/ml would give complete selection of non transformed plants both at embryogenic mass and at plantlet stage. But presuming that such a high concentration at the initial stage itself might cause death and malformation of transformed tissue, following strategy was adopted for selection of transformants. Embryogenic mass after co cultivation with *Agrobacterium* in the regeneration medium were subjected to selection in three stages with increasing concentration of antibiotic: initially at 25 µg/ml for 3-5 weeks followed by 50 µg/ml for next 30 days. At plantlet stage selection was done at 100 µg/ml.

Optimization of carbenicillin and cefotaxime concentration to control *Agrobacterium* over growth

Cefotaxime at 25 µg/ml and above or carbenicillin at 50 µg/ml and above were able to control the *Agrobacterium* growth from co cultivated embryo or embryogenic mass up to 30 days while carbenicillin at 25 µg/ml failed to control the *Agrobacterium* overgrowth.

Embryo along with surrounding micropylar tissue did not show any primary somatic embryogenesis in presence carbenicillin while reduced somatic embryogenesis was seen in presence of cefotaxime. But embryogenic mass showed cyclic secondary embryogenesis differentiation in to plantlets with same concentration of antibiotics indicating that embryogenic mass can be used as ideal explants for *Agrobacterium* mediated transformation experiments in black pepper. Embryogenic mass treated with cefotaxime at 100 µg/ml enhanced somatic embryo proliferation and also was able to control *Agrobacterium* over growth. Hence this concentration of cefotaxime was used for all further experiments.

Transformation using GUS construct and analysis of transformants

The co cultivated embryogenic mass in selection medium (25 µg/ml of kanamycin) under darkness for 3 – 5 weeks produced some vigorous growing points. An average of 10 such kanamycin resistant growing points was obtained per g of embryogenic mass. Each of these growing points was considered as independent transformation. These growing points were removed onto the same medium with a higher kanamycin concentration (50 µg/ml) for

another 30 days for further proliferation in to an embryo cluster. At this stage transfer to liquid SH medium supplemented with 3% sucrose promoted faster growth and differentiation of embryos in to plantlets. Kanamycin was avoided from the medium at this stage. An average of 32 fully developed plantlets (3.2 plantlets/growing point) were regenerated in the liquid SH medium. About 90% plantlets were rooted without any additional treatments in liquid culture. In the final selection medium, (WPM with 100 µg/ml kanamycin), an average of 23 plantlets per g of embryogenic mass was obtained. Plantlets that retained green colour even after 30 days of final kanamycin selection were transferred to WPM without kanamycin for further growth of plantlets. The survived (non-bleached) plantlets were hardened in green house of which an average of nine plantlets per g of embryogenic mass could be established in the green house. All transformed and hardened putative transformants showed normal growth and development with uniform appearance like any normal black pepper plants.

Confirmation of transgenicity

Assay for GUS activity

The results showed uniform blue colour in transformed embryogenic mass in contrast to the colourless negative control after overnight incubation with the substrate suggesting that the GUS gene was transcribed from the transformed embryogenic mass and resulted in the synthesis of functional enzyme. The colourless nature of non transformed embryogenic mass after staining indicate that there is no detectable endogenous GUS activity in non transformed control embryogenic mass.

PCR analysis of transformed plants

PCR analysis of 108 kanamycin resistant lines revealed the specific predicted amplification products of 980 bp and 660 bp with NPT II and GUS gene specific primers, respectively while no such bands were visible in non transformed plantlets. This indicated the presence of both the linked genes NPT II and GUS as a single T-DNA in the transformed genome. Out of 108 plants analyzed 92 were found positive in PCR.

Dot blot and Southern hybridization

Results of dot blot hybridization showed presence of NPT II gene in 62 of the putative transgenic plants out of 74 plants tested. Southern analysis confirmed the stable integration of the NPT II gene in six of the putative transgenics out of 17 plants tested indicating that 35%

of the dot blot positive plants were positive in Southern as well. One to four bands were seen per sample in the Southern blot.

3.2 Development of gene constructs using CMV and PYMoV sequences in plant transformation vectors

ORF III region of *Piper yellow mottle virus* (PYMoV) in sense and antisense orientation

Presence and orientation of recombinant clone in *E. coli* strain DH5α and *Agrobacterium* strain EHA 105 was confirmed by PCR and restriction analysis. The sense orientation clone in pBI121 was identified by PCR primer designed for the CaMV 35S promoter region as forward primer and reverse primer of ORF III region as reverse primer that gave an expected size of 539 bp. Similarly antisense clone in pBI 121 was identified by PCR using primer designed for the CaMV 35S promoter region as forward primer and forward primer of ORF III region as reverse primer that gave an expected size of 539bp. Results obtained from PCR were further confirmed by restriction analysis of recombinant plasmids using restriction enzyme *Bam*H1. Gel electrophoresis of restricted product showed a band of 409 bp (ORF III gene) and a 14.8 kb vector band. The sense and antisense constructs were designated as pBI121PYMoVS and pBI121PYMoVAS respectively.

Cucumber mosaic virus (CMV) coat protein in sense orientation

The presence of recombinant pBI 121 containing CMV CP gene (sense orientation) in *E. coli* (Strain DH5α) and *Agrobacterium* strain EHA 105 was confirmed by PCR and restriction. PCR was performed using two combinations of primer pairs, CaMV 35S forward + CMVCP reverse and CaMV 35S forward + CMVCP forward. The former primer pair gave an expected amplicon of 780 bp but the latter combination did not give any amplicon. Results obtained from PCR were further confirmed by restriction analysis of recombinant plasmid using the restriction enzymes *Bam*H1 and *Sac*I. Gel electrophoresis of restricted product showed a band of 657 bp (CMVCP gene) and a 12.9 kb vector band confirming the recombinant nature of the plasmid. The sense construct of CMV was designated as pBI121CMVS.

3.3 Transformation of embryogenic mass with PYMoV sense (pBI121PYMoVS) and antisense (pBI121PYMoVAS) constructs and analysis of transformants

Embryogenic mass was transformed with *Agrobacterium* harbouring pBI121PYMoVS, pBI121PYMoVAS constructs and plantlets were regenerated under antibiotic selection. In the case of pBI121PYMoVS construct, an average of 27 fully developed plantlets (2.7 plantlets/growing point) were regenerated in the liquid SH medium. About 90% plantlets were rooted without any additional treatments in liquid culture. In the final selection (WPM with 100 µg/ml kanamycin), an average of 16.7 plantlets per g of embryogenic mass was obtained. These plantlets were hardened in green house of which an average of 10 plantlets per g of embryogenic mass could be established. In pBI121PYMoVAS construct an average of 33.7 fully developed plantlets (3.37 plantlets/growing point) were regenerated in the liquid SH medium. In the final selection (WPM with 100 µg/ml kanamycin), an average of 25.3 plantlets per g of embryogenic mass was observed. These plantlets were hardened in green house of which an average of 8.7 plantlets per g of embryogenic mass could be established in the green house with a hardening efficiency of 60% and 34%. PCR was performed using two sets of primers. One set primer was specific for kanamycin region while the other set was specific for transgene gave an amplicon of approximately 980 bp in positive plants where as the transgene specific primers gave an amplicon of 539 bp in case of both sense and antisense constructs. In the case of pBI121PYMoVS construct all the 30 plants screened were positive in PCR where as in pBI121PYMoVAS construct out of 78 plants screened, 62 plants tested as positive in PCR.

In the case of pBI121PYMoVS construct out of 30 plants subjected to dot blot, 24 plants gave positive signal (80%) where as out of 44 plants in pBI121PYMoVAS construct screened, 38 were found to be positive (86%). Plants that gave intense spots in dot blot assay were taken for Southern hybridization. In pBI121PYMoVS construct, two plants showed positive signals out of 10 screened where as four out of seven tested showed positive signal in the case of pBI121PYMoVAS construct. In sense construct single copy of transgene integration was observed in both the plants while in antisense construct one to two integration events were seen. Out of four Southern positive plants in antisense construct one showed two copy and remaining three plants showed single copy integration.

3.4 Transformation of embryogenic mass with *Cucumber mosaic virus* coat protein (CMVCP) sense construct and analysis of transformants

Agrobacterium carrying the recombinant binary vector, pBI121CMVS were used for transforming black pepper. Transformation using pBI121CMVS construct gave an average of 34 fully developed plantlets (3.5 plantlets/growing point) in the liquid SH medium. About 90% plantlets were rooted without any additional treatments in liquid culture. In the final selection (WPM with 100 µg/ml kanamycin), an average of 26 plantlets per g of embryogenic mass was obtained. These plantlets were hardened in green house of which an average of 18 plantlets per g of embryogenic mass could be established in the green house. All the survived plants were hardened in green house with a hardening efficiency of 70%.

PCR was performed using two sets of primers specific for kanamycin gene and transgene. Kanamycin specific primers gave an expected amplicon of around 980 bp in positive plants where as the transgene specific primers gave an amplicon at 780 bp. Out of 109 plants screened, 104 plants were found positive in PCR. Selected PCR positive plants were subjected to dot blot assay. Out of 80 plants subjected to dot blot, 43 plants gave positive signals. Samples that gave intense spots in dot blot assay were subjected to Southern hybridization. All nine plants tested in Southern hybridization showed positive signal indicating integration of transgene with the host genome. Of the nine Southern positive plants, three plants showed single copy integration while in the remaining four plants showed two copies and two plants showed four copies of transgene integration.

FUTURE LINE OF WORK

Transgenic black pepper plants expressing ORF III region of *Piper yellow mottle virus* in sense and antisense orientation and *Cucumber mosaic virus* coat protein in sense orientation produced in this study are maintained in insect proof glass house. After proper establishment, these plants need to be subjected to challenge inoculation with PYMoV and CMV respectively. Presence of virus within the plants may be monitored by visual symptoms and PCR along with inoculated non transgenic control plants. Resistant lines if any should be subjected to repeated subculturing to ensure the stability of the transgene.

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